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Pravastatin Does Not Prevent Antiphospholipid Antibody Effects On Human First Trimester Trophoblast Function

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PRAVASTATIN DOES NOT PREVENT ANTIPHOSPHOLIPID ANTIBODY EFFECTS ON HUMAN FIRST TRIMESTER TROPHOBLAST FUNCTION

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ABSTRACT

Women with antiphospholipid syndrome (APS) have circulating antiphospholipid antibodies (aPL) and are at risk for recurrent pregnancy loss and late pregnancy complications due placental dysfunction. Recent research has demonstrated that aPL directly alter human first trimester trophoblast function by up-regulating inflammatory cytokines, limiting cell migration, and altering angiogenic factor production. Due to their anti-inflammatory properties, Statins have been tested both *in vitro,* using human trophoblast, and *in vivo,* using a mouse model, for the treatment of APS-associated pregnancy complications and preeclampsia. *In vivo* mouse studies showed that statins prevent aPL- mediated fetal loss, whereas *in vitro* human studies suggest that statins, like pravastatin may compromise normal trophoblast function and viability. Therefore, the objective of this study was to test the hypothesis that pravastatin prevents the effects of aPL-on human first trimester trophoblast function. The human first trimester trophoblast cell line, HTR8, and first trimester trophoblast primary cultures were incubated with or without an aPL in the presence or absence of pravastatin. Cytokine and angiogenic factor secretion were measured by ELISA and multiplex analysis. Cell migration was measured using a colorimetric two-chamber migration assay. Pravastatin significantly augmented the aPL-induced up-regulation of IL-8, IL-1β and sEndoglin secretion by HTR8 cells, but had no effect on aPL-induced up-regulation of VEGF, PlGF and GRO-α. Furthermore, pravastatin alone limited basal HTR8 cell migration, and did not mitigate the adverse effect of aPL on trophoblast migration. However, Pravastatin

had no effect on aPL-mediated changes in primary first trimester trophoblast function. These findings demonstrate that pravastatin does not prevent the effects of aPL antibody on first trimester trophoblast cell function, and so may not be beneficial as a therapeutic for pregnant APS patients. However, it did not have any negative effects on basal primary cell function and, therefore, may be safe to use in patients at high risk for preeclampsia.

ACKNOWLEDGEMENTS

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TABLE OF CONTENTS

INTRODUCTION

Antiphospholipid syndrome (APS), also known as Hughes syndrome, is a systemic autoimmune disease that is diagnosed when a person with laboratory evidence of persistent circulating antiphospholipid (aPL) antibody presents clinically with vascular thrombosis and/or pregnancy morbidity [1, 2, 3, 4]. Persistent circulating aPL refers to the presence of aPL on at least two separate occasions that are at least 6 weeks apart [1]. The pregnancy morbidities in pregnant APS patients vary and include recurrent fetal loss (rate can be up to 90% when no specific treatment is given [5]), as well as late term complications such as preeclampsia, HELLP Syndrome (Hemolytic anemia, Elevated Liver enzymes and Low Platelet count), premature birth and intrauterine growth restriction (IUGR) [6]. However, the pregnancy morbidities required for the diagnosis of APS are history of unexplained recurrent fetal loss, or premature birth secondary to preeclampsia or any other feature suggestive of placental insufficiency [2]. In addition to obstetric complications, APS patients in general are at significant risk of vascular thrombosis; hence it is an alternative clinical requirement for diagnosis of the disease. In fact, overall, thrombotic complications are the main source of complications in APS patients [7] as they are 3-10 times more likely to have thrombosis than people without aPL antibodies [1]. Vascular thrombosis can be both arterial (especially cerebral vessels) and venous (especially the deep veins of the lower extremities).

APS is caused by a group of heterogenous autoantibodies which, although they are called antiphospholipid antibodies, actually bind to phospholipid-binding proteins such as Annexin V, Protein C, Prothombin and beta 2-glycoprotein 1 (β_2GPI) [2, 3, 8]. The clinically significant aPL used in the diagnosis of APS are either ELISA-detected IgG or IgM anticardiolipin (aCL) and/or anti-β_2GPI antibodies or a functional assay for lupus antigoagulant (LA) that determines its anticoagulant activity against β_2GPI and prothrombin [2]. The LA test is most commonly used for diagnosis. These antibodies are present in 7- 25% of women with recurrent fetal loss [2, 3]. Though lupus anticoagulant is one of the antibodies used in diagnosis of APS, and one third of patients with systemic lupus erythematous (SLE) have aPL and may in fact meet the criteria for APS, it is important to note that SLE is a separate entity from APS; there are specific and different set of criteria for diagnosis of SLE [9]. APS can be present in the absence of any other autoimmune disease (called primary APS in such scenario), or in the presence of another autoimmune disease such as SLE, in which case it is designated as secondary APS.

Of the known phospholipid-binding proteins, β_2GPI is the most clinically significant in the pathology of APS and all the afore mentioned aPL antibodies (LA, aCL and anti-β_2GPI) can bind to it. However, one can have LA and aCL without β_2GPI activity because LA can also bind to prothrombin, and while β_2GPI is a cofactor for binding of aCL in APS, there is also β_2GPI-independent aCL that is associated with infectious process [2]. Beta 2 glycoprotein 1 is a highly glycosylated protein with a

lysine-rich domain that interacts with and is immobilized when it binds to negatively charged membrane phospholipids such cardiolipin and phosphatidyl serine [2, 8]. The formation of an aPL-β_2GPI complex increases the affinity of β_2GPI for membrane phospholipid [2].

While most cells will only bind exogenous β_2GPI on their cell surface under pathologic, stimulatory or apoptotic conditions, when the inner negatively charged phospholipids become exposed onto the outer leaflet of the plasma membrane [8, 10, 11, 12], the trophoblast (cells of the placenta) is unusual in that it normally expresses these anionic phospholipids on its cell surface [8, 13, 14, 15]. This is due to trophoblast cell's high level of tissue remodeling involving high levels of proliferation and differentiation as part of normal placentation [8]. For review, normal placentation begins with the blastocyst adhering to the uterine wall. This is followed by invasion of the endometrium by a subset of trophoblast cells called cytotrophoblasts, the trophoblast stem cell, which undergoing proliferation to regenerate the functional cells that constitute the placenta [16]. These cytotrophoblasts can take two differentiation routes: 1) to undergo fusion to form the outer syncytiotrophoblast layer of the placenta; and 2) invading deep into the maternal decidual tissue, and here differentiating into extravillous trophoblast cells. These extravillous trophoblast cells then proceed to invade the maternal spiral arteries and remodel these vessels, differentiating into endovascular trophoblast cells and replacing the maternal endothelial cells. This transformation of the

maternal vasculature results in increased blood flow into the placental intervillous space. The presence of anionic phospholipids on the outer leaflet of the trophoblasts has been linked to their abilities to undergo their normal functions, including fusion to form the syncytium [2, 15, 17]. Therefore due to the presence of anionic phospholipids on its outer leaflet of the cell membrane, the trophoblast can bind exogenous β_2GPI on its cell surface under normal physiological conditions. Additionally, trophoblast normally produces its own β_2GPI, and *in vivo*, there is evidence that β_2GPI localizes to the surface of the extravillous trophoblast cells that invade the decidua, and to the syncytiotrophoblast cells that are in direct contact with maternal blood [8]. These reasons explain the tropism of aPL for the placenta [2, 4, 8] and is further supported by the laboratory finding that passive transfer of human aPL into pregnant mice leads to its disappearance from peripheral circulation and accumulation in the placenta [2]. The fact that the placenta is a major target for aPL partly explains why pregnancy complications associated with altered placental development and function occur in women with APS.

Apart from impairing trophoblast fusion and differentiation into giant multinucleated cells [2], binding of aPL can decrease the production of normal pregnancy hormone production [2, 12] and alter cytokine production [2,8]. These can partially explain the observation of insufficient placentation (evidenced by reduced trophoblast invasion) and limited spiral artery transformation in APS, similar to what is seen in preeclampsia [8, 18]. Further, the process of decidualization in a

normal pregnancy includes recruitment of specialized population of maternal immune cells, which eventually compose about 70% of cells in the uterus during pregnancy [19]. These cells, consisting of specialized natural killer cells (~75%), some monocytes and dendritic cells (~15%) and regulatory T cells (~15), are not inflammatory and do not participate in cellular cytotoxicity; rather, they play an immunoregulatory role, which is essential for a successful pregnancy as they are able to coexist with foreign cells and enhance maternal tolerance [19]. Unlike normal in normal pregnancy, the decidua in APS patients has inflammatory immune cell (macrophages and neutrophils) infiltration [20, 21, 22].

Given the propensity for vascular thrombosis in APS patients, pregnancy failure in APS was initially attributed to thrombosis at the maternal fetal interface [3, 8]. Hence the original method of therapy for the high rates of miscarriages was anticoagulation with heparin with or without aspirin. To date, the standard of care for obstetrical APS includes either low dose aspirin before conception and throughout pregnancy up to 4 months postpartum, or low molecular weight heparin (LMWH) plus low dose ASA during pregnancy [2]. However, both clinical observations and experimental findings have called this basis of treatment into question. Histological studies have shown that intravascular or intervillous blood clots are not commonly found in samples of placentas from APS patients with miscarriage [23]. Further, clinically, while treatment with heparin increases live birth rates, it does not prevent late term complications of APS such as preeclampsia [3, 5, 24, 25, 26]. Moreover, its

efficacy is controversial and is yet to be confirmed in a randomized controlled trial [27, 28]. Hence, despite treatment, pregnant APS patients remain high-risk obstetric cases throughout gestation. Additionally, studies have raised questions about the safety of these therapeutics, in particular low molecular weight heparin (LMWH). Carroll *et al* found that *in vitro*, LMWH independently up-regulated first trimester trophoblast expression of the potent anti-angiogenic factor, soluble fms-like tyrosine (sFlt) [29], which is associated with preeclampsia [30, 31, 32, 33, 34]. Other researchers have reported this finding both in vitro [35, 36] and in vivo in pregnant women treated with heparin [35, 37]. Moreover, Han *et al* showed that LMWH also induced an increase in basal GROα secretion by first trimester trophoblasts [36]. These observations and findings prompted further investigation into the true pathologic mechanism(s) involved in APS, as this would direct better the identification of novel therapeutics and thus, the management of APS patients.

Similar to what is observed in endothelial cells, where binding of aPL to β_2GPI on the surface of the endothelial cells leads to induction of a pro-inflammatory state [1, 38], recent studies now strongly demonstrate a primary role for inflammation at the maternal-fetal interface, rather than thrombosis, in APS-associated pregnancy pathology. Studies have shown histological signs of acute and chronic inflammation in the placental beds of samples of miscarriages in APS patients [21, 22]. Additionally, studies with animal models of APS have shown local and/or systemic elevation of inflammatory markers such as TNFα and tissue factor level [20, 39].

Further, complement C3 deposition in the decidua has been observed, and blocking of the complement pathway or tissue factor prevented aPL-mediated pregnancy failure and associated inflammation [40, 41,42, 43]. And, as mentioned earlier, both placentas from animal models of APS and human tissues have shown inflammatory immune cell infiltration [20, 21]. Most recently, studies from the Abrahams lab, have shown that aPL markedly enhances human first trimester trophoblast production of pro-inflammatory cytokines, such as interleukin 8 (IL 8), Interleukin 1β (IL1-β), monocyte chemoattractant protein 1 (MCP-1) and GRO alpha (GROα), via TLR4/MyD88 dependent pathway [44]. Further, the lab has illustrated that aPL inhibit trophoblasts constitutional secretion of interleukin 6 (IL 6), is a cytokine that is associated with trophoblast invasiveness [45, 46], leading to decreased STAT3 activity and subsequently trophoblast migration [47]. Indeed, IL-6 deficient mice exhibit impaired implantation and reduced litter size [45, 46]. Therefore, the trophoblast expression of IL 6 is important for normal trophoblast migration. Hence impairment of IL-6 production by aPL, may lead to failure of the trophoblast to effectively perform one of its basic functions in pregnancy. Also, aPL perturbs angiogenic factor profile by up-regulating vascular endothelial growth factor (VEGF), placental growth factor (PlGF) and soluble endoglin [29]. These findings, combined with aforementioned *in vivo* animal studies, plus the dearth of thrombosis in the histology of placenta from miscarriages in APS patients, has led to a widespread acceptance that non-thrombotic, inflammatory mechanisms are the main causes of defective placentation in APS. Even the observed partial efficacy of

heparin supports this notion as studies have shown that heparin possesses anti-inflammatory property due to its ability to inhibit complement activity [3, 41]. In one study, treatment of pregnant APS mice with pure anticoagulants such as fondaparinux and hirudin did not prevent aPL-induced pregnancy loss, whereas treatment with unfractionated heparin and low molecular weight heparin suppressed complement activity and increased live birth rate [41]. Hence the observed efficacy of heparin is likely due to its anti-inflammatory properties, not its anticoagulant property. However, heparin cannot reverse all effects of aPL on trophoblast function and as aforementioned, might exacerbate some [29, 36]. Hence, there continues to be growing interest towards finding alternative treatments for the pregnant APS patients.

In the pursuit of better treatment therapy, 143-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA) reductase inhibitors (statins) have been proposed for use in preventing APS-associated pregnancy complications. In addition to lowering cholesterol, statins have been shown to possess anti-inflammatory properties. The anti-inflammatory properties of statins, as observed mostly in atherogenesis, include: a decrease in immune cell infiltration of tissues; a decrease in adhesion molecules such as ICAM-1, VCAM-1, E-selectin and P-selectin; a decrease in chemoattractant proteins such as MCP-1 and IL-8; a decrease in pro-inflammatory transcription factors such as NFκB; a decrease in pro-inflammatory enzymes such as COX-2; a decrease in pro-inflammatory cytokines such as TNFα, IL-1β, IL-6; and a decrease in inflammatory

serum markers such as CRP [48, 49]. Even though statins are currently contraindicated in pregnancy due to risk of birth defects [50], the proposal for its use in APS has been based on the finding that pravastatin can prevent fetal loss in aPL-treated mice [51]. However, the reported benefit of pravastatin in mouse model of APS has yet to be confirmed in a human model of APS. Moreover, other studies have reported that statins negatively affect human first trimester trophoblast by decreasing migration and proliferation, and inducing apoptosis [52, 53, 54].

Therefore, in light of these conflicting data, we decided to test the main hypothesis that **pravastatin could prevent the effects of aPL on the trophoblast without affecting basal trophoblast function**. Thus, the objective of this current study was to determine whether pravastatin could be used for the treatment of aPL-mediated pregnancy complications. In order to test this, specific aims of this project were to investigate:

Aim 1: The effects of pravastatin on human first trimester trophoblast cell viability

Aim 2: The effects of pravastatin on human first trimester trophoblast cytokine and angiogenic factor secretion in the absence and presence of aPL.

Aim 3: The effects of pravastatin on human first trimester trophoblast cell migration the absence and presence of aPL.

MATERIALS AND METHODS

Source and Maintenance of Trophoblast cells

The human first trimester extravillous trophoblast cell line, HTR8, and primary trophoblast cultures isolated from first trimester placentas (8-12wks gestation) were used in these studies. The HTR8 cells, immortalized by SV40 [55], were a gift to the lab from Dr. Charles Graham (Queens University, Kingston, ON, Canada).

HTR8 cells were cultured in growth media, prepared by supplementing RPMI 1640 media (from Gibco) with 10% fetal bovine serum, 0.01% of 10mM Hepes, 0.01% of 0.1mM MEM non-essential amino acids, 0.01% 1mM sodium pyruvate, and 0.01% of 100nm penicillin/streptomycin. The cells were grown at 37˚C/5% CO_2 in a sterile 75cm^2 polystyrene cell culture flask. When fully confluent, cells were split and then either plated for experiments, froze in liquid nitrogen for future lab work or maintained by continued culture.

To split confluent cells for an experiment, 3-5ml of 0.05% Trypsin was added in the culture flask containing the cells. Trypsin was then activated by brief incubation of the cells at 37˚C until the cells had detached. Equal volume of growth media was added and the mixture transferred to a sterile 10ml polypropylene conical tube, centrifuged at 21˚C at 3000rpm at room temperature for 10mins and the pellet then re-suspended in 5-10ml of growth media. Cells were then counted and plated for experimentation as described below.

For primary cultures, first trimester placentas were obtained from elective terminations of normal pregnancies performed at Yale-New Haven Hospital. The use of patients samples was approved by Yale University's Human Investigation Committee. Ms. Mulla prepared tissue specimens as follows: Cells were washed with cold Hanks Balanced Salt Solution to remove excess blood. They were then scraped from the membranes, transferred to trypsin-EDTA digestion buffer and incubated at 37˚C for 40 minutes with shaking. The mixture was then passed through a nylon strainer and then layered over Lymphocyte Separation Media and centrifuged at 2000rpm for 25 minutes. The cellular interface containing the trophoblast cells was collected and re-suspended in D-MEM with D-valine supplemented with 10% normal human serum and cultured in 5% CO_2 at 37˚C.

Antiphospholipid antibodies

The mouse IgG1 anti-human β_2GPI monoclonal Ab (mAb), IIC5, was used in these studies. This antibody was produced by Larry W. Chamley (department of Obstetrics and Gynecology, University of Auckland, New Zealand), under sterile conditions, and was filter-sterilized prior to use. IIC5 was cloned from mice immunized with purified human β_2GPI, and has been previously characterized [56]. Like human aPL, IIC5 binds β_2GPI, but only when it is immobilized on a suitable negatively charged surface, such the phospholipids, cardiolipin, phosphatidyl serine, or irradiated polystyrene [57]. IIC5 binds to first trimester trophoblast cells

and similar to patient-derived polyclonal aPL, it alters trophoblast function by inducing pro-inflammatory cytokine expression and perturbing angiogenic factor balance [29, 44].

Cell viability studies

Trophoblast cell viability was determined using the CellTiter 96 viability assay. Cells were plated in wells of a 96-well plate at $1x10^4$ cells per well in growth media, at total volume of 100µL, and cultured at 5% CO_2 and 37˚C.When cells became 70% confluent media was replaced with 50µL of serum-free Opti-MEM media (Invitrogen), and cultured for another 4hrs. Treatments were prepared at double the target concentrations and 50µL of treatments were added to the wells, bringing down the treatments to the final concentrations of CPT at 4µM and pravastatin at 0.1, 1, 2.5 and 5µg/ml. All treatments were prepared in Opti-MEM. Cells were then incubated for 72hrs at 37˚C and afterwards, 20µL CellTiter 96™ substrate was added to all wells. Cells were the incubated for an additional 2hrs at 37°C and optical densities were read at 490 nm. All samples were assayed in triplicate, and cell viability was presented as a percentage of the untreated control.

Cytokine and angiogenic factor secretion studies

Cytokine and angiogenic factor secretion were assessed using enzyme linked immunosorbent assay (ELISA) and multiplex analysis. Prior to this, HTR8 trophoblast cells were plated at 6 x 10^4cells/ml in total volume of 2ml of growth

media, in sterile 35mm petri dishes and cultured in growth media in similar conditions as above. When the cells were 70% confluent, the growth media was replaced with 2ml of OptiMEM and cells incubated for 4hrs at 37°C. OptiMEM was then replaced with treatments that consisted of IIC5 (aPL) at 20µg/ml (in OptiMEM) in the presence and absence of pravastatin at either 0.5 or 2.5µg /ml in OptiMEM. After 72 hours at 37°C, cell-free supernatants were collected by centrifugation at 2000rpm at 4°C for 5 minutes and stored at -80˚C until analysis was performed.

For primary human first trimester trophoblast, the cells had heterogeneous growth rates since they were collected from different patient samples. As such, when plated, the cells in different plates attained 70% and 100% confluence at different times. Additionally, since we cannot culture them as long as the immortalized cell lines, we performed the experiments as soon as some of the petri dishes had 70% confluent cells. Therefore following treatments, just as in the case of HTR8 cells, and collection of supernatants, the cells were lysed and the total proteins collected. Bicinconinic assay (BCA) analysis was done to normalize the protein levels in the different treatment groups and to appropriately interpret the ELISA results. Total protein, was collected thus: After collecting the supernatant cold 1xPBS was added to the dishes and cells scraped off the dish using an autoclaved cell scraper. The mixture was then transferred to 15ml conical tube and centrifuged at 1500rpm for 10mins at 4°C. The pellet was re-suspended with 50µL of lysis buffer (containing 0.02% PIC and PMSF), vortexed and incubated on ice for 20mins. Afterwards, the lysate was

centrifuged at 13000rpm for 15mins at 4°C and supernatant stored at -40°C until ready for BCA assay.

At the time of analysis of supernatants from either HTR8 cells or primary trophoblasts, supernatants were thawed at room temperature and evaluated the concentrations of interleukin 8 (IL-8), vascular endothelial growth factor (VEGF), placenta growth factor (PlGF), soluble fms-like tyrosine kinase-1 (sFlt-1) and soluble Endoglin (sEndoglin) using R&D systems ELISA kits and following the manufacturer's instructions. The levels of IL-8, IL-1β, GRO-α and VEGF were also determined by multiplex analysis of the supernatants using the BioPLex assay with detection and analysis using the Luminex 100 IS system.

Cell migration studies

To assess trophoblast migration, a two chamber colorimetric assay was employed. The top chambers were made of 8µm pore size cell culture inserts, while the lower chambers were 24-well tissue culture plates. The lower chamber was filled with 800µL of OPtiMEM and the top chamber was seeded with $1x10^5$cells HTR8 cells, which were suspended in 200µL of treatments (media alone, aPL, pravastatin, or both). Following a 48hr incubation, inserts were removed and trophoblast migration across the membrane was determined using the QCM 24-Well Colorimetric Cell Migration assay according to the manufacturer's direction (Chemicon International). Briefly, the filters on the top chamber was removed and transferred into wells containing 250µl of cell stain. This process stains the migrated cells, which were

trapped in the pores of the filter, to stain. Afterwards, excess stain was removed by dipping the bottom of the filter into a beaker of distilled water and transferring filter into a clean well. 200□l of extraction buffer was then added to the wells and cells left to gently rock at 1.5rpm at room temperature for 15mins. 50□l of the resulting colored mixture was transferred to a 96-well plate and optical densities read in triplicate at 560 nm. A 100% migration control consisted of 1 x 10^5 HTR8 cells placed directly in the 24-well plate without any insert. Observed optical density values were measured using a BioRad plate reader, and were compared to the 100% migration control to determine the relative percent migration.

Statistics

All experiments were performed at least three times. I produced all of the data involving the HTR8 cell lines (cell viability, cytokine and angiogenic factor studies, and migration studies) as well as two sets of data for primary trophoblasts cell viability studies and one set of data for IL-8 levels. Ms. Mulla obtained the remaining data for primary trophoblast viability studies, cytokine and angiogenic factor production in the presence of pravastatin. Similarly, with the help of Dr. Abrahams, I performed the statistical analysis for data for HTR8 cells while Dr. Abrahams performed the statistical analysis of data for the primary trophoblast cells. Data are expressed as mean ± standard deviation (S.D.) of pooled experiments. Statistical significance (p<0.05) was determined using either the one-way Anova with the Bonferroni correction, or the paired student's t-test.

RESULTS

Effects of pravastatin trophoblast viability

Previous studies have reported that statins can prevent human trophoblast proliferation and induce trophoblast apoptosis [52]. Therefore, the first objective of this study was to determine the effects of pravastatin on trophoblast cell viability under our laboratory conditions. Thus, the HTR8 cell line and primary trophoblast cultures were treated for 72hrs with either no treatment (NT), camptothecin (CPT, 4μM), which serves as a positive control for cell death, or pravastatin at 0.1, 1, 2.5 or 5 μg/ml. As shown in Figure 1A, treatment with pravastatin at the highest dose of 5μg/ml significantly decreased the viability of the first trimester trophoblast cell line, HRT8, by 22.9 ± 33.1% (p<0.05). As expected our positive control, CPT, also significantly decreased HTR8 cell viability by 78% ± 4.8% (p<0.001). At the lower doses of pravastatin (0.1 – 2.5μg/ml), there was no significant effect on HTR8 trophoblast cell viability (Figure 1A). In contrast, pravastatin, at all doses, did not have any affect on the viability of primary human first trimester trophoblast cell (Figure 1B).

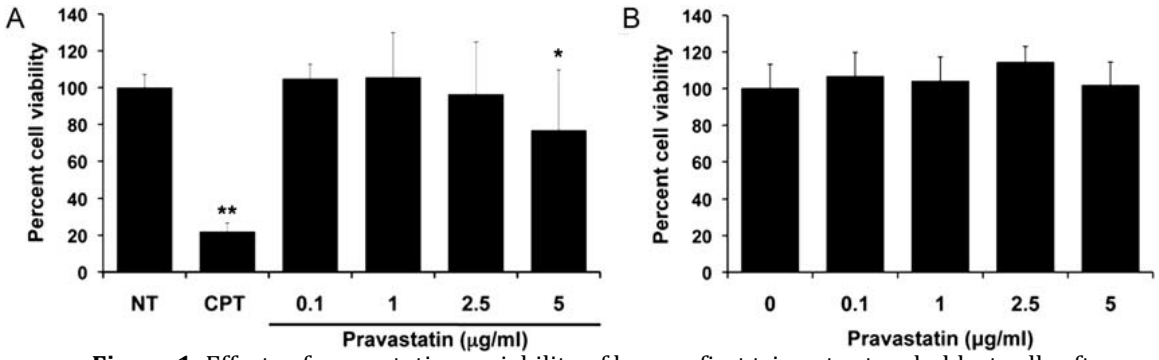

Figure 1: Effects of pravastatin on viability of human first trimester trophoblast cells after incubation for 72hrs. (A) HTR8 trophoblast cell line (B) primary human first trimester trophoblasts. *P<0.05; **P<0.001; NT = no treatment, CPT = 4μM of camptothecin.

Due to the effect of the high dose pravastatin on HRT8 cells, all subsequent experiments were performed at lower doses that did not affect cell viability. Thus, for all other experiments, a low dose of 0.5μg/ml and a high dose of 2.5μg/ml was used.

Effects of Pravastatin on aPL-induced inflammation on trophoblasts.

We have previously demonstrated that the mouse anti-human β_2GPI mAb, IIC5, and patient-derived polyclonal aPL, with β_2GPI activity, induce a human first trimester trophoblast inflammatory response by up-regulating the cell's secretion of IL-8, IL-1β and GRO-α [36, 44]. Therefore, we investigated whether pravastatin could counter these effects of aPL. Thus HTR8 cells were treated for 72hrs with or without the anti-β_2GPI Ab, in the presence of either media, pravastatin at 0.5μg/ml or pravastatin at 2.5μg/ml after which IL-8, IL-1β and GRO-α secretion was measured. As shown in figure 2A high and low doses of pravastatin had no significant effect on

basal IL-8 or GRO-α secretion by HTR8 cell lines, but did significantly elevate basal IL-1β at the high dose (NT groups in figure 2A). Surprisingly, the presence of pravastatin at both high and low doses significantly augmented the anti-β_2GPI mAb-induced up-regulation of IL-8 secretion by HTR8 cells (anti-β_2GPI mAb groups in figure 2A). Additionally, low dose pravastatin significantly augmented the anti-β_2GPI mAb up-regulation of IL-1β secretion. Pravastatin had no significant effect on the up-regulation of GRO-α induced by the anti-β_2GPI mAb on HTR8 cells.

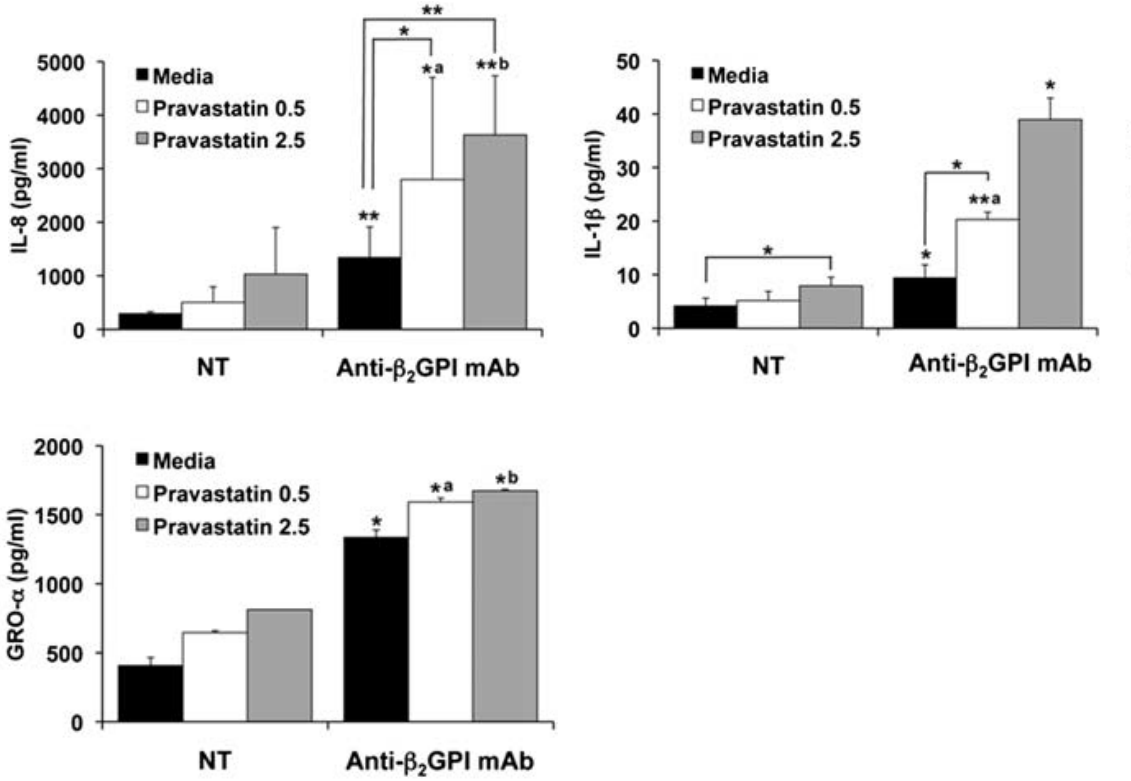

Figure 2A: Pravastatin augments aPL-mediated secretion of IL-8, IL-1β, and GROα by human first trimester trophoblast cell line, HTR8. Treatment groups received 20μg/ml of anti-β2GP1 mouse Ab (IIC5) and 0.5 or 2.5μg/ml of pravastatin and all (including control) were incubated for 72hrs. NT = No treatment; *P<0.05; **P< 0.01; a = P<0.05 relative to NT; b = P<0.01 relative to NT.

In order to validate our findings, we next tested whether pravastatin had similar effects on primary human first trimester trophoblasts. Interestingly, in contrast to our findings using the HTR8 cell line, neither high nor low dose pravastatin altered the basal cytokine production in primary human first trimester trophoblasts (figure 2B). Also, pravastatin had no effect on the aPL-induced up-regulation of pro-inflammatory cytokines, IL-8, IL-1β and GRO-α, secreted by primary trophoblast cells (figure 2B).

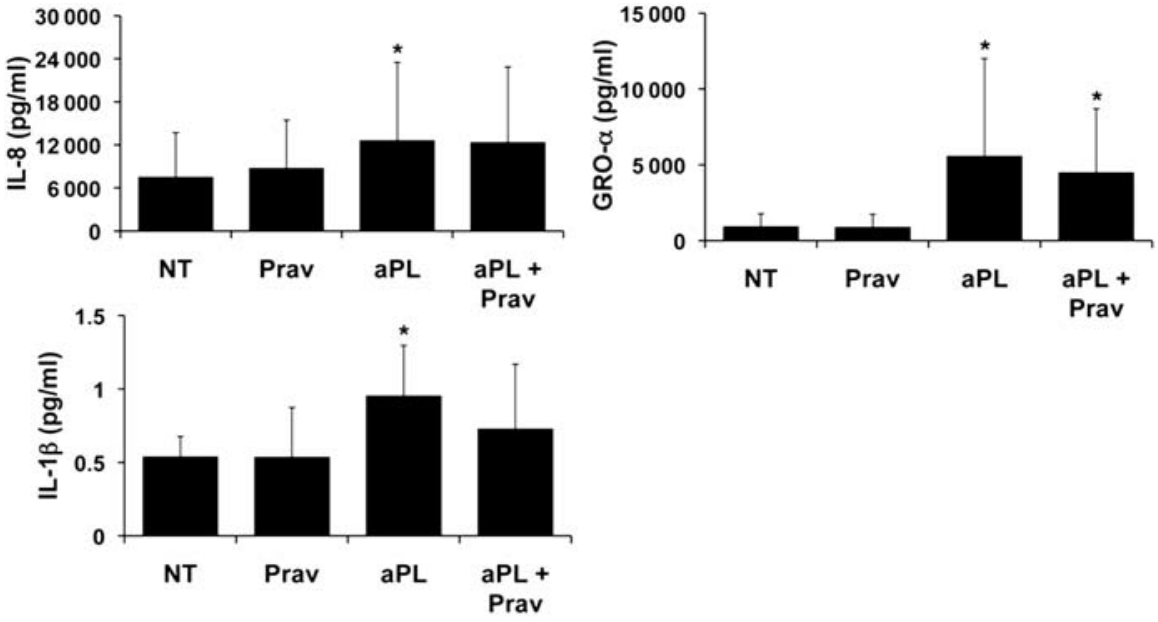

Figure 2B: Effects of pravastatin on aPL -mediated secretion of IL-8, IL-1β, and GROα by primary human first trimester trophoblast. Treatment groups received 20μg/ml of anti-β2GP1 mouse Ab (IIC5) and 0.5 or 2.5μg/ml of pravastatin and all (including control) were incubated for 72hrs. NT = No treatment; *P<0.05 and **P< 0.01 relative to NT.

Effects of pravastatin on aPL-induced anti-angiogenic factor response in trophoblasts.

We have recently demonstrated that the mouse anti-β_2GPI mAb, IIC5, and patient-derived polyclonal aPL, alter angiogenic factor profile by up-regulating human first trimester trophoblast cell secretion of VEGF, PlGF and soluble endoglin [29, 36]. Therefore, one of the objectives of this study was to determine whether pravastatin impacts these angiogenic factor alterations caused by aPL. To test this, HTR8 cells were treated for 72hrs with or without the anti-β2GPI Ab, in the presence of either media, pravastatin at 0.5μg/ml or pravastatin at 2.5μg/ml after which VEGF, PlGF, soluble endoglin and sFlt-1 levels were measured. As shown in figure 3A, pravastatin alone had no effect on the basal production of VEGF, PlGF or sEndoglin by HTR8 cells (NT groups in Figure 3A). But, at the high dose of 2.5μg/ml, basal sFlt-1 secretion was significantly increased (p <0.01). Consistent with our prior studies, treatment of HTR8 cells with anti-β_2GPI mAb led to up-regulation of VEGF, PlGF and soluble endoglin with down-regulation of sFlt-1. Pravastatin slightly, yet significantly, reduced the ability of the anti-β_2GPI mAb to increase HTR8 VEGF and PlGF secretion (anti-β_2GPI mAb groups in Figure 3A). However at both high and low doses, pravastatin significantly augmented anti-β_2GPI mAb-induced up-regulation of sEndoglin secretion by HTR8 (p< 0.01 and p<0.05 respectively).

Since we had observed differential effects of pravastatin on basal and aPL-mediated changes in pro-inflammatory cytokine secretion by HTR8 cells and primary

trophoblasts, we were curious about the effects of pravastatin on basal and aPL-mediated changes in aniogenic factor secretion by primary human first trimester trophoblasts. Apart from a significant decrease in basal sEndoglin level, pravastatin did not alter basal angiogenic factor production by primary first trimester trophoblasts. Furthermore, similar to the pattern in pro-inflammatory cytokine production, pravastatin did not reverse any of the aPL-mediated changes in angiogenic factor profiles of primary human first trimester trophoblasts (figure 3B).

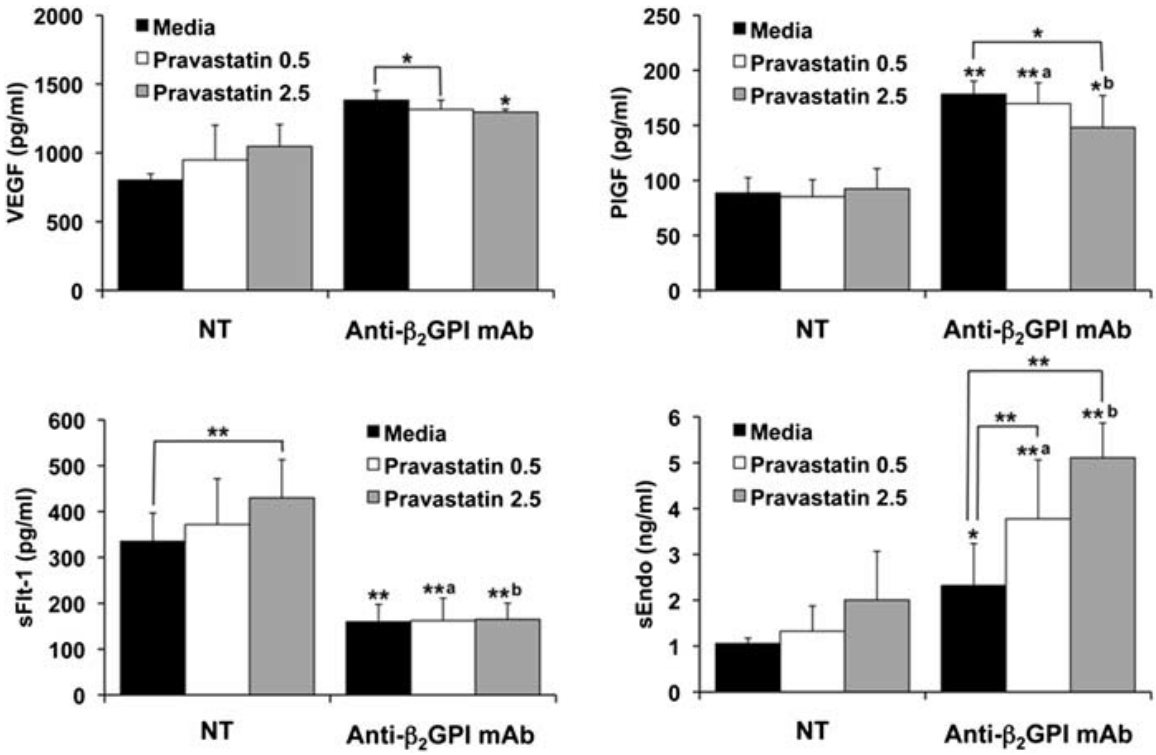

Figure 3A: Pravastatin augments aPL-induced up-regulation of soluble endoglin (sEndo) by human first trimester trophoblast cell lines, HTR8. Treatment groups received 20μg/ml of anti-β2GP1 mouse Ab (IIC5) and 0.5 or 2.5μg/ml of pravastatin and all (including control) were incubated for 72hrs. NT = No treatment; *P<0.05; **P< 0.01; a = P<0.05 relative to NT; b = P<0.01 relative to NT.

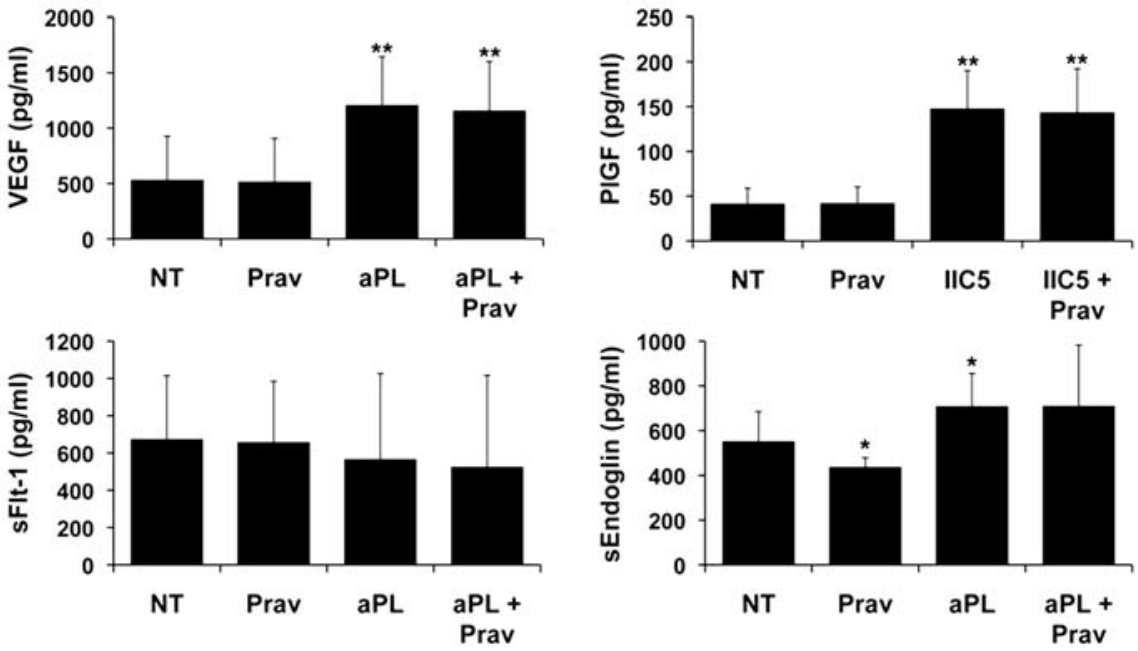

Figure 3B: Figure 2B: Effects of pravastatin on aPL-mediated secretion of angiogenic factors by primary human first trimester trophoblast. Treatment groups received 20μg/ml of anti-β2GP1 mouse Ab (IIC5) and 0.5 or 2.5μg/ml of pravastatin and all (including control) were incubated for 72hrs. NT = No treatment; *P<0.05 and **P< 0.01 relative to NT.

Pravastatin does not reverse aPL inhibition of trophoblast migration.

In addition to inducing a pro-inflammatory and anti-angiogenic profile, anti-β_2GPI mAb limit first trimester trophoblast migration [47]. Since the ability to migrate is one of the essential functions of the trophoblast and impaired migration and invasion predisposes to fetal loss in APS, it was important to us to assess whether pravastatin could mitigate this impaired migration caused by aPL. Therefore, HTR8 cells were treated for 48hrs with or without the anti-β_2GPI Ab, in the presence of either media, pravastatin at 0.5μg/ml or pravastatin at 2.5μg/ml after which cell migration was determined using colorimetric two-chamber migration assay. As

shown in Figure 4, pravastatin alone at the low dose of 0.5μg/ml slightly but significantly reduced basal HTR8 cell migration ($p < 0.01$). At either doses, pravastatin had no effect on the inhibition of trophoblast migration induced by the anti-β_2GPI mAb.

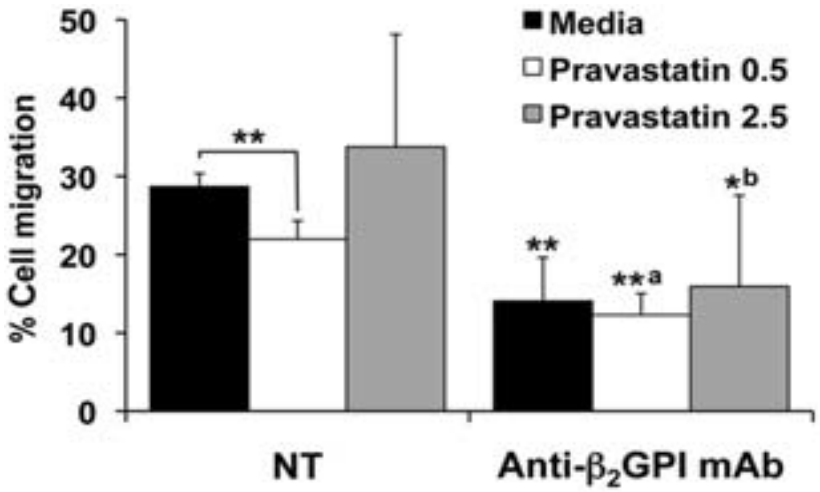

Figure 4: Pravastatin did not reverse aPL-antibody mediated decrease in migration of human first trimester trophoblast cell line, HTR8. Treatment groups received 20μg/ml of anti-β2GP1 mouse Ab (IIC5) and 0.5 or 2.5μg/ml of pravastatin and all (including control) were incubated for 72hrs. NT = No treatment; *P<0.05; **P< 0.01; a = P<0.05 relative to NT; b = P<0.01 relative to NT.

DISCUSSION

The majority (about 90%) of fetal losses seen in APS patients occurs during the first trimester, usually before 14 weeks of gestation [5]. Since aPL are present at the time of implantation, this suggests that the pathology in obstetric APS may evolve during the first trimester of pregnancy, similar to what is thought for preeclampsia [58, 59]. Therefore, understanding this pathology as it occurs early in pregnancy would help in the prevention of both the fetal loss and later term complications in APS. For this reason, we have chosen to study APS pathology and the efficacy of novel therapeutics *in vitro* using first trimester trophoblast cells.

While women with APS are routinely treated with LMWH during pregnancy, clinical and experimental studies have highlighted the potential lack of efficacy of this regimen, as well as potential problems resulting from the treatment [27, 28, 29, 35, 36, 37]. Consequently, there is a need for alternative preventative strategies in the management of pregnant women with APS. Recent studies in mice have demonstrated that the statin, pravastatin, prevents the onset of symptoms of preeclampsia brought about either by the overexpression of the anti-angiogenic factor human sFlt-1 [32, 33, 34] or the genetic background [60, 61], suggesting that it could be beneficial in prevention of preeclampsia.

Preeclampsia is a common adverse pregnancy outcome in women with APS [6, 62, 63]. In this patient population, it can have early-onset and particularly be severe leading to high rate of preterm delivery [62]. Furthermore, similar to what is seen in preeclampsia, the histology of the placenta in APS shows shallow invasion of the decidua by the trophoblast, as well as poor remodeling of maternal spiral arteries [16, 23]. However, the mechanism for this observed phenomenon is likely different from preeclampsia. In APS, there is premature stimulation of cytotrophoblast proliferation and syncytial fusion leading to depletion of cytotrophoblasts [16]. Since the cytotrophoblast is the trophoblastic stem cell, there is ultimately reduced number of stem cell pool to form syncytiotrophoblasts (the most important component of materno-fetal barrier) and extravillous trophoblasts that invade the decidua and maternal vessels. There is no consensus on the cause of the premature stimulation of cytotrophoblast proliferation but with the large body of literature showing inflammation in APS, inflammation could play a role in this observed phenomenon. Due to the similar end results in placental morphology, the association between preeclampsia and APS, and the noted anti-inflammatory properties statins, researchers have tested the efficacy of pravastatin in APS model. Pravastatin was shown to prevent aPL-mediated pregnancy loss in mice [51, 64]. These findings of the effects of pravastatin in mouse models of classical preeclampsia and APS have led to an interest in the use of statins to treat pregnancy complications, such as preeclampsia and those associated with APS [51, 64]. Indeed, a randomized placebo-controlled clinical trial on statins for the prevention of early-

onset preeclampsia, **St**atins to **Am**eliorate Early Onset **P**re-eclampsia (StAmP Trial), is currently underway [65]. Since the impact of statins have yet to be tested in a human model of aPL-associated pregnancy problems, and studies using normal human placental explants have raised concerns over the use of statins during pregnancy [52, 53, 54], we sought to determine whether statin could prevent aPL-mediated effects using a human trophoblast *in vitro* system.

We used pravastatin in our studies because it is the statin that has been shown to be least associated with risk of fetal anomaly due to low lipophilicity [66]; since it does not easily crossing the placenta, it is thought to remain on the maternal side of the placenta. Since the objective of this study was to find out whether pravastatin could be used for the treatment of aPL-mediated pregnancy complications, low diffusion through the placenta was an important feature as any favorable finding can be extrapolated to an *in vivo* human model without fear of damage to the fetus, while still potentially benefiting placentation by minimizing the inflammation at the maternal side of the placenta and countering the effects of aPL on the placenta. In addition to having low permeability through the placenta, pravastatin was used because it has been shown to have beneficial effects in previous experimental studies in mice [32, 33, 34, 51]. Based on prior studies using pravastatin [54] and other statins [52], we were able to select a dose range that we considered appropriate to address the question in this study. In our preliminary experiments we found that while pravastatin had no adverse effect on the viability of primary first trimester

trophoblast cultures, pravastatin at 5μg/ml, reduced the viability of human first trimester trophoblast cell line HTR8, an observation also made by other groups using human first trimester placental explants [52, 54]. Hence, we used pravastatin at concentrations (0.5μg/ml and 2.5μg/ml) that did not induce cell death. At these lower doses, we found that pravastatin modestly enhanced sFlt-1 and IL-1β secretion and reduced the migratory capacity of HTR8 trophoblast. Our findings are in keeping with the observation by others, demonstrating that statins reduce trophoblast cell migration and outgrowth from first trimester explant cultures [52, 53].

We also failed to observe unequivocal beneficial effects of pravastatin on HTR8 trophoblast exposed to the anti-β_2GPI mAb. Pravastatin was unable to prevent the up-regulation of the pro-inflammatory cytokines IL-8, IL-1β, and GRO-α; the induction of sEndoglin; or the reduction in cell migration upon exposure to aPL antibody. Pravastatin did, however, reduce aPL antibody-induced VEGF and PlGF secretion. While this reached significance, these effects were modest and unlikely to have physiological consequences. Moreover, this finding does not support the observation in the mouse model of preeclampsia where pravastatin increases PlGF expression [34].

Where we did see a marked effect by pravastatin was in its ability to augment aPL-induced secretion of IL-8, IL-1β and sEndoglin by the HTR8 trophoblast cell line.

Indeed studies using immune cells have shown that statins can enhance LPS-induced inflammation, including IL-8 and IL-1β production [67, 68, 69]. Since aPL-induced IL-8 and IL-1β is mediated by TLR4 [44], the mechanism by which pravastatin enhances the trophoblast inflammatory response to aPL may be similar. However, when the effect of pravastatin was tested using primary human first trimester trophoblast cultures, we found no positive or negative effects on either basal or aPL-stimulated cytokine and angiogenic factor production.

The reason for the difference in observations between the trophoblast cell line and primary cells may be because the primary cultures are a more heterogeneous population. Alternatively, since we have observed similar effects of the aPL on both the primary trophoblast and the trophoblast cell line in our previous studies [29, 44], our results may indicate that pravastatin has differential effects on the two types of culture. Indeed, the augmentation of aPL-induced IL-1β, IL-8 and sEndoglin secretion by pravastatin in the cell line may be a reflection of pravastatin's ability to slightly elevate the cell line's basal production of these factors; an observation not seen in the primary cultures.

Based on our observations we can conclude that while pravastatin may not be overtly detrimental to the trophoblast in the absence of aPL in terms of cytokine and angiogenic factor production, alone it still might impair placental cell function by limiting migration, which is critical for normal placentation; and it fails to protect

the trophoblast from aPL effects. Thus, our findings might be taken to not support the use of pravastatin in APS patients. Why this is in contrast to the *in vivo* studies showing that pravastatin prevents aPL-induced pregnancy loss may simply be a consequence of species differences, with different pharmacokinetics in each species. Alternatively, it might be a result of methodology. In vivo, significant maternal blood flow to the villous tissues begins around weeks 10-12; before this, there is low oxygen concentration in the intervillous space and it enables trophoblast to rapidly proliferate while protected from free oxygen free radical [16]. This hypoxic condition is not represented in our experimental system as we performed our incubations at normoxic conditions. Therefore, another possibility for the differences between the cell types could be a result of the oxygen concentration used. Furthermore, in the mouse model of aPL-induced pregnancy failure, pravastatin was administered prior to aPL exposure, whereas in our *in vitro* model, trophoblast cells were treated simultaneously with aPL and pravastatin. Moreover, the mouse model explains the pathogenesis of APS-associated pregnancy failure and the success of pravastatin in preventing this is based on its ability to prevent aPL-induced neutrophil tissue factor and PAR-2 expression [51, 64], rather than the direct effects of aPL on the trophoblast, as in our model [29, 36, 44, 47]. So in our system, which is relatively free of those agents, we observed a differential effect. Thus, we can conclude that in our study, pravastatin fails to prevent the direct effect of aPL on the placenta and trophoblast function.

Finally, our findings demonstrate that, *in vitro*, pravastatin does not prevent the effects of anti-β_2GPI Abs on human first trimester trophoblast cell function and, therefore, may not be beneficial as a therapeutic for pregnant APS patients. Though there is a study in the mouse model of APS showing reduced miscarriage rates due to pravastatin treatment, whether pravastatin can prevent aPL-associated late-term pregnancy complications is unclear at this time because this efficacy is yet to be reproduced in other studies; and prior to our study, there was no study testing pravastatin in human APS model. Additionally, though the litters produced in the mouse model after treatment with pravastatin appeared normal, we do not yet know the long-term effects of this treatment on the off-spring. So more studies are needed to address these issues and determine the effectiveness of pravastatin in the prevention of aPL-associated pregnancy complications in humans. In the current state of care of APS patients, there is use of heparin and/or aspirin, which while they do not prevent late term complications, they are non-teratogens and to some extent do significantly reduce miscarriages. So while current research aims to improve treatment options in APS, at this point the risk of using pravastatin, a potential teratogen, outweighs its benefit in APS. In contrast to APS, there is more data that can be viewed as support of the benefit of pravastatin in models of preeclampsia. However, while there is abnormal placentation in both classical preeclampsia and APS, it is worth noting that both are separate entities with different underlying mechanisms; therefore efficacy of pravastatin in classical preeclampsia may not directly translate into efficacy for preeclamptic APS patients.

In summary, this research study has demonstrated that pravastatin augments the aPL-induced inflammation and anti-angiogenic profile in the first trimester trophoblast cell line, but has no effect on aPL-induced responses in primary trophoblast cells. These findings demonstrate that pravastatin does not prevent the effects of aPL antibody on first trimester trophoblast cell function, and so may not be beneficial as a therapeutic for pregnant APS patients. However, with respect to the normal functions of the primary human first trimester trophoblast cell, particularly their innate ability to migrate, produce cytokines necessary for its function (such as IL 6), and produce angiogenic factors under resting, basal conditions, our study did not reveal any overt negative effects of pravastatin. With this finding and the fact that certain studies have illustrated its benefits in preeclampsia, independent of aPL [32, 33, 34], one might argue that pravastatin might be considered for use in non-APS patients at high risk for preeclampsia. While studies suggest that this could be a beneficial option in preeclampsia, the risk of fetal anomaly with statin use still remains a significant possible adverse outcome. Therefore, since delivery of the placenta is a definitive treatment for preeclampsia, and in many cases can be accomplished without dangers of the lifetime sequel of prematurity, it may be worthwhile to follow that route for mild to moderate preeclampsia. Pravastatin use can then be reserved for the few cases of severe preeclampsia where the fetus is significantly premature and delivery would place the fetus at high risk of lifetime complications of prematurity. Further, even in such cases, it is difficult to

extrapolate, from our study, the appropriate dose that would be required for treatment of preeclampsia since the only documented use of statins in pregnancy is old literature about inadvertent use for treatment of hyperlipidemia. Also, unlike in our studies, statins are typically clinically dosed on a fixed milligram amount regardless of patient weight. Additionally though not well studied at this point, the physiology of pregnancy could alter the pharmacokinetics of pravastatin and such changes may not be fully captured in *in vitro* studies like ours. Therefore more studies, such as the on-going randomized controlled clinical trial [65] would provide such helpful information. Meanwhile, there are limited *in vivo* studies using pravastatin to prevent aPL-mediated pregnancy complications and so more research is needed in that area. Future studies by us will be directed towards elucidating the mechanism through which pravastatin augments aPL-mediated responses in the cell line.

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